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# Protection against UVR Involves MC1R-Mediated Non-Pigmentary and Pigmentary Mechanisms *In Vivo*

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Individuals with red hair and fair skin due to MC1R gene variants are at higher risk of cutaneous neoplasia, consistent with MC1R having a role in photoprotection. The exact reasons for greater UVR susceptibility as a result of compromised MC1R function are unclear, but hypotheses include reduced photoprotection due to less eumelanin, pheomelanin-induced phototoxicity, and lower protection by "non-pigmentary" MC1R effects. To determine how MC1R photoprotects, an *in vivo* hairless MC1R model containing $Mc1r^{-/-}$ albino, $MC1R^{+}Mc1r^{-/-}$ albino, $Mc1r^{-/-}$ pigmented, and $MC1R^{+}Mc1r^{-/-}$ pigmented mice was generated. After single doses of UVR, no significant differences in epidermal cyclobutane pyrimidine dimers or sunburn cell (SBC) formation were observed between pigmented and albino groups. However, after repeated UVR exposure, the number of p53 clones in albino skin was significantly elevated when this was null for MC1R. Furthermore, in the absence of functional MC1R, fewer p53 clones were observed in pigmented than in albino skin. The results indicate that MC1R protects by a combination of pigmentary and non-pigmentary effects *in vivo* and that when MC1R function is compromised the melanin type in skin is still protective against UVR.

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## INTRODUCTION

Exposure of skin to UVR results in DNA damage, clonal evolution of abnormal cells, and ultimately skin cancer, with Caucasians at higher risk of UVR damage than Asian and Negroid subjects (Melnikova and Ananthaswamy, 2005; Yamaguchi *et al.*, 2006). Individuals most susceptible to these harmful effects are those with ''Celtic'' pigmentary characteristics, i.e., red hair and fair skin (Gandini *et al.*, 2005; Pelucchi *et al.*, 2007). Skin and hair color is determined by the relative amounts of brown-black eumelanin and red-yellow pheomelanin, synthesized by melanocytes in these tissues (Ito and Wakamatsu, 2003). The red hair/fair skin phenotype frequently arises from genetic variants at the *melanocortin 1 receptor* (*MC1R*) locus, which encodes for a receptor on the surface of melanocytes (Valverde *et al.*, 1995; Healy *et al.*, 2000; Sulem *et al.*, 2007). ''Loss of function'' *MC1R* variants, which are responsible for fair skin, result in reduced receptor numbers at the cell membrane and/or significantly compromised intracellular signaling, resulting in an imbalance of melanin synthesis toward that of pheomelanin (Beaumont *et al.*, 2005, 2007).

It is acknowledged that certain *MC1R* variants can significantly reduce receptor function and that red hair and fair skin is the null phenotype for MC1R in humans (Healy *et al.*, 2001; Beaumont *et al.*, 2008); however, the mechanism(s) responsible for higher susceptibility of red-haired fair-skinned subjects to UVR damage remain to be clarified. It is likely that reduced eumelanin content in fair skin results in less photoprotection, but because pheomelanin can generate free radicals *in vitro* after UVR, pheomelanin phototoxicity has also been considered a causative factor (Chedekel *et al.*, 1978; Harsanyi *et al.*, 1980; Wenczl *et al.*, 1998). Support for the latter view has been provided by the observation of greater UVR damage, as detected by TUNEL positivity, in hair follicles of mice with a pheomelanic coat (Takeuchi *et al.*, 2004). However, many studies have reported that the association of *MC1R* variants with skin dysplasia/cancer persists after controlling for skin type/tanning ability and hair color, suggesting that loss of MC1R function may have ''non-pigmentary'' consequences (Bastiaens *et al.*, 2001; Box *et al.*, 2001; Landi *et al.*, 2005; Liboutet *et al.*, 2006). Furthermore, *MC1R* variants can alter DNA repair/apoptotic responses *in vitro* after UVR and influence behavior of melanoma cells independently of effects on pigmentation (Robinson and Healy, 2002; Böhm *et al.*, 2005; Kadekaro *et al.*, 2005). Consequently, the extent to which less eumelanin, relatively more pheomelanin, and non-pigmentary MC1R-mediated effects are responsible for greater UVR-induced damage in

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*Abbreviations: CPD, cyclobutane pyrimidine dimer; MC1R/Mc1r, melanocortin 1 receptor; MEdD, minimal edema dose; SBC, sunburn cell; TPC, TUNEL-positive cell*

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fair-skinned individuals with functionally compromised *MC1R* variants, and conversely the mechanisms by which MC1R photoprotects, are at present unclear.

We have generated an ''albino and pigmented hairless MC1R'' model to analyze the mechanisms underlying MC1R photoprotection *in vivo*. Our results suggest that MC1R offers significant protection against UVR by a combination of MC1R-mediated pigmentary and non-pigmentary effects, and that the pheomelanin/eumelanin ratio in skin secondary to loss of MC1R function is photoprotective rather than phototoxic.

## RESULTS

### *In vivo* hairless MC1R model

To analyze how MC1R protects against UVR, we generated a mouse model in which each litter contained animals of four different genotype–phenotype groups: $Mc1r^{-/-}$ albino (A), $MC1R^{+}Mc1r^{-/-}$ albino (MA), $Mc1r^{-/-}$ pigmented (P), and $MC1R^{+}Mc1r^{-/-}$ pigmented (MP) (Figure 1). After an initial hair cycle, in which hair was white (A and MA), yellow (P), or black (MP), the skin became hairless, enabling UVR studies to be undertaken on adult mice aged 6–12 weeks. The stratum corneum shields against UVR and the number of epidermal cell layers can influence penetration of UVR (Diffey, 1983); therefore, adult dorsal skin samples ($n = 6$ per group) were examined to ensure that the stratum corneum and epidermal thicknesses were similar in the A, MA, P, and MP groups. No significant differences in these parameters were observed between the four groups (Figure 2).

Immunostaining of whole-mount epidermal sheets from adult dorsal skin using an anti-tyrosinase-related protein-1 antibody showed the presence of epidermal melanocytes in the A, MA, P, and MP mice (Figure 2). Masson-Fontana staining of dorsal skin identified eumelanin in the epidermis of MP animals, but no epidermal eumelanin in the other groups. High-performance liquid chromatography analysis for pyrrole-2,3,5-tricarboxylic acid and 4-amino-3-hydroxyphenylalanine was conducted on epidermal sheets taken from the mid-back. This identified eumelanin and pheomelanin in the pigmented animals, with mean levels of eumelanin 272 ng mg$^{-1}$ and pheomelanin 8.9 ng mg$^{-1}$ (eumelanin/pheomelanin ratio 30.6) in the MP group and eumelanin 129 ng mg$^{-1}$ and pheomelanin 13.5 ng mg$^{-1}$ (eumelanin/pheomelanin ratio 9.6) in the P group (Table 1). In the chronically irradiated pigmented skin, the levels of eumelanin and pheomelanin were similar to those of unirradiated skin (MP, eumelanin 257 ng mg$^{-1}$ and pheomelanin 9.6 ng mg$^{-1}$; and P, eumelanin 140.8 ng mg$^{-1}$ and pheomelanin 12.5 ng mg$^{-1}$), with eumelanin/pheomelanin ratios of 26.8 and 11.3, respectively, in the MP and P mice. Although these absolute amounts of melanin are lower than that in human skin, the eumelanin/pheomelanin ratios are comparable to that in humans (Hennessy *et al.*, 2005). Levels

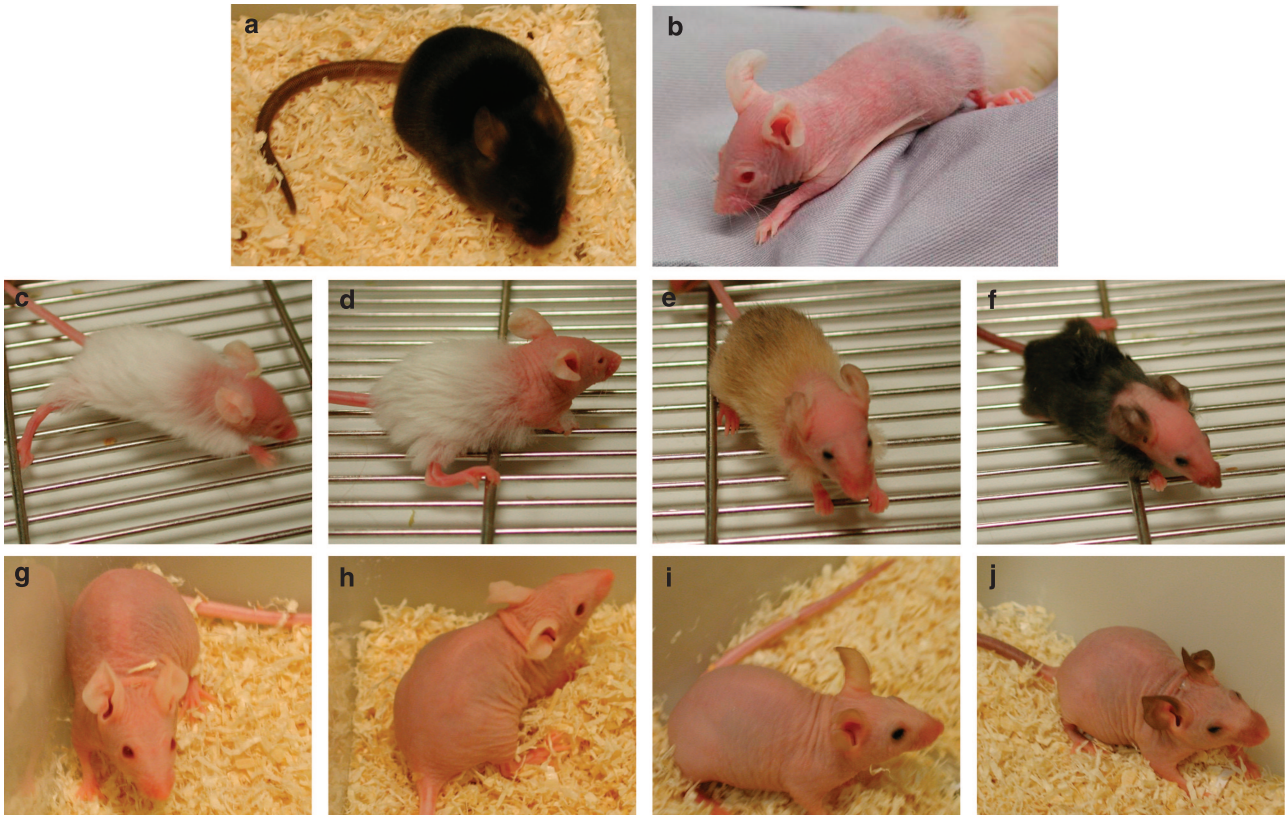


**Figure 1. Development of *in vivo* melanocortin 1 receptor (MC1R) model.** (**a**, **b**) $MC1R^{+}Mc1r^{-/-}$ haired mice (**a**) were crossed with albino Skh:hr-1 hairless mice (**b**) to generate an *in vivo* MC1R model containing $Mc1r^{-/-}$ albino (A), $MC1R^{+}Mc1r^{-/-}$ albino (MA), $Mc1r^{-/-}$ pigmented (P), and $MC1R^{+}Mc1r^{-/-}$ pigmented (MP) animals. (**c**, **g**) A, (**d**, **h**) MA, (**e**, **i**) P, and (**f, j**) MP mice during (**c**–**f**) and after (**g**–**j**) first hair cycle.

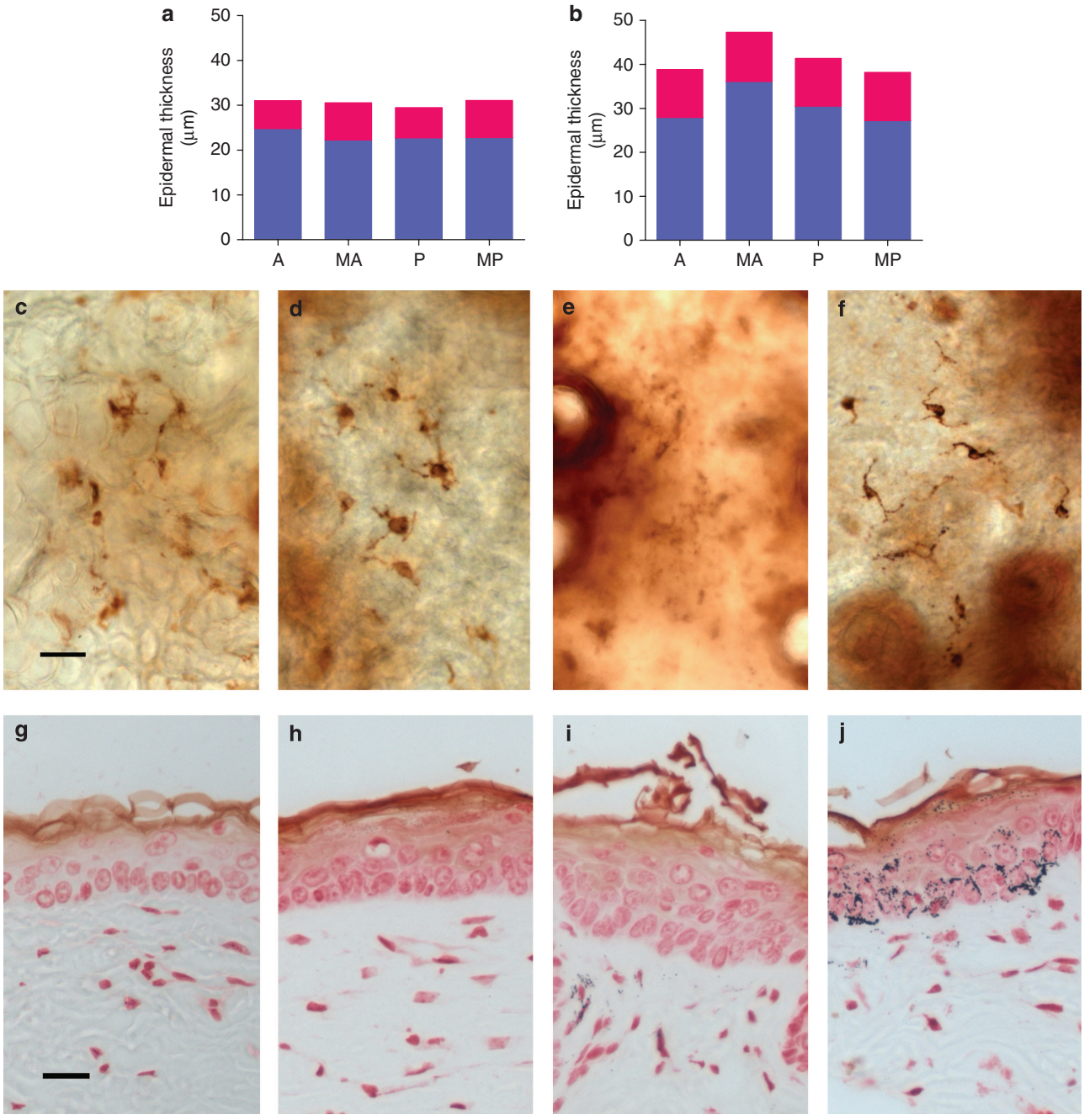


**Figure 2. Stratum corneum/epidermal thickness, melanocytes, and eumelanin in A, MA, P, and MP mice.** (**a**, **b**) Thickness of nucleated epidermis (blue) and stratum corneum (pink) in naive mice was similar in each group (**a**). After 6 weeks of repeated UV, epidermal thickness increased to similar extent in all four groups (**b**), $n=6$ mice per group. (**c**–**f**) Tyrosinase-related protein-1 staining of epidermal sheets from (**c**) $Mc1r^{-/-}$ albino (A), (**d**) $MC1R^{+}Mc1r^{-/-}$ albino (MA), (**e**) $Mc1r^{-/-}$ pigmented (P), and (**f**) $MC1R^{+}Mc1r^{-/-}$ pigmented (MP) mice shows interfollicular epidermal melanocytes in all four groups. (**g**–**j**) Masson-Fontana staining of sections from (**g**) A, (**h**) MA, (**i**) P, and (**j**) MP mice showing eumelanin in epidermis of MP, but not in A, MA, and P mice. Scale bars = 20 μm.

of melanin were below the detection limits in the unirradiated and chronically irradiated skin of the albino mice.

### Responses of naive and acclimatized skin to single-dose UV irradiation

Naive mice, not previously UV irradiated, were exposed to 5.15 kJ $m^{-2}$ and 20.6 kJ $m^{-2}$ (equivalent to 0.5 and 2 minimal edema doses (MEdD)) of UVR. Exposure of skin to UVR results in formation of cyclobutane pyrimidine dimers (CPDs), which are subsequently repaired by nucleotide excision repair (Mouret *et al.*, 2006; Nijhof *et al.*, 2007); therefore, dorsal skin from A, MA, P, and MP animals ($n=8$ mice per group) was examined for CPD at 30 minutes and at 24 hours after UVR. No significant difference in numbers of

**Table 1. Levels of eumelanin and pheomelanin in epidermis of pigmented mice[1]**

| Unirradiated skin | Eumelanin | Pheomelanin | Eumelanin/ pheomelanin ratio[2] | Mean eumelanin/ pheomelanin ratio | *P*-value[2] |
|---|---|---|---|---|---|
| P | 76 | 17.1 | 4.4 | 9.6 | 0.028 |
| P | 110 | 8.3 | 13.3 | | |
| P | 265 | 16.8 | 15.8 | | |
| P | 64 | 11.8 | 5.4 | | |
| MP | 101 | 6.2 | 16.26 | 30.6 | |
| MP | 334 | 6.8 | 48.8 | | |
| MP | 381.5 | 13.6 | 28.1 | | |
| | | | | | |
| *Chronically irradiated skin* | | | | | |
| P | 129 | 7.6 | 17 | 11.3 | 0.032 |
| P | 155 | 9.2 | 16.8 | | |
| P | 140 | 9.1 | 15.4 | | |
| P | 139 | 24.1 | 5.8 | | |
| MP | 302 | 6.6 | 45.8 | 26.8 | |
| MP | 224 | 14.6 | 15.3 | | |
| MP | 236 | 5.7 | 41.4 | | |
| MP | 267 | 11.6 | 23 | | |

Abbreviations: MP, $MC1R^{+}Mc1r^{-/-}$ pigmented; P, $Mc1r^{-/-}$ pigmented.
[1]Levels of melanin were below detection limits in the skin of albino animals.
[2]*P*-values represent comparison of eumelanin/pheomelanin ratio between P and MP animals.

CPD-positive epidermal nuclei was observed in any of the four groups at 30 minutes after 0.5 MEdD (Figure 3) and 2 MEdD (data not shown). At 24 hours, fewer epidermal nuclei were CPD positive and intensity of CPD staining was reduced, but no significant difference was observed between the four groups at either UVR dose at this time point (Figure 3).

To examine for differences between the four groups in UVR-acclimatized skin, mice were irradiated with 0.2 MEdD, 5 days per week for 6 weeks. During this process, the skin of the MP mice became darker (tanned), presumably because of a redistribution of melanin in their skin (Yamaguchi *et al.*, 2006); the P animals developed a light yellow hue to their skin, whereas the albino mice remained pale. The stratum corneum and epidermis also thickened, but no significant differences in these were detected between the four groups (Figure 2). After 4 days (to allow repair of any CPDs from this low-dose acclimatization schedule), mice were irradiated with identical UVR doses to those administered to naive skin (5.15 kJ $m^{-2}$ and 20.6 kJ $m^{-2}$). Again, no significant variation in the numbers of epidermal CPD-positive nuclei between mouse groups was noted at 30 minutes and at 24 hours after UVR (Figure 3). Furthermore, no disparities in the intensity of CPD staining between the four groups were detected in any of the above experiments.

No evidence for a difference in epidermal proliferation, as assessed by Ki67 expression, was noted between the four groups at 24 hours after UVR (data not shown). However, cell death can serve as a protective mechanism after UVR, such that cells with irreparably damaged DNA undergo apoptosis, thus preventing clonal growth of abnormal cells. Therefore, we examined for TUNEL-positive cells (TPCs) and sunburn cells (SBCs) in naive and acclimatized skin at 24 hours after UVR. There was some amount of variation in the numbers of TPCs and SBCs in the mice, but no significant difference in TPCs in naive skin and in SBCs in naive and acclimatized skin between groups (Figure 4). In the acclimatized skin, there were fewer TPCs in the MA and MP groups when compared with A and P groups, respectively, suggesting that MC1R may have protected slightly against UVR and/or reduced UVR-induced cell death. There were also more TPCs in the P than the A mice (and MP mice), consistent with the finding by Takeuchi *et al.* (2004) that pheomelanin may increase UVR-induced TPC death, but these comparisons within our data were not statistically significant.

### Pigmentary and non-pigmentary effects of MC1R on formation of p53 clones

Chronic exposure of skin to UVR results in development of clones of keratinocytes immunopositive for p53, with many of these containing p53 mutations (varying from 29 to 64% in individual studies), especially in exons 5–8 (Jonason *et al.*, 1996; Tabata *et al.*, 1999; Bäckvall *et al.*, 2004; Kramata *et al.*, 2005; Rebel *et al.*, 2005). Similar p53 mutations have been identified in non-melanoma skin cancers, consistent with the view that UVR-induced p53 gene damage, if inadequately repaired, allows proliferation of abnormal

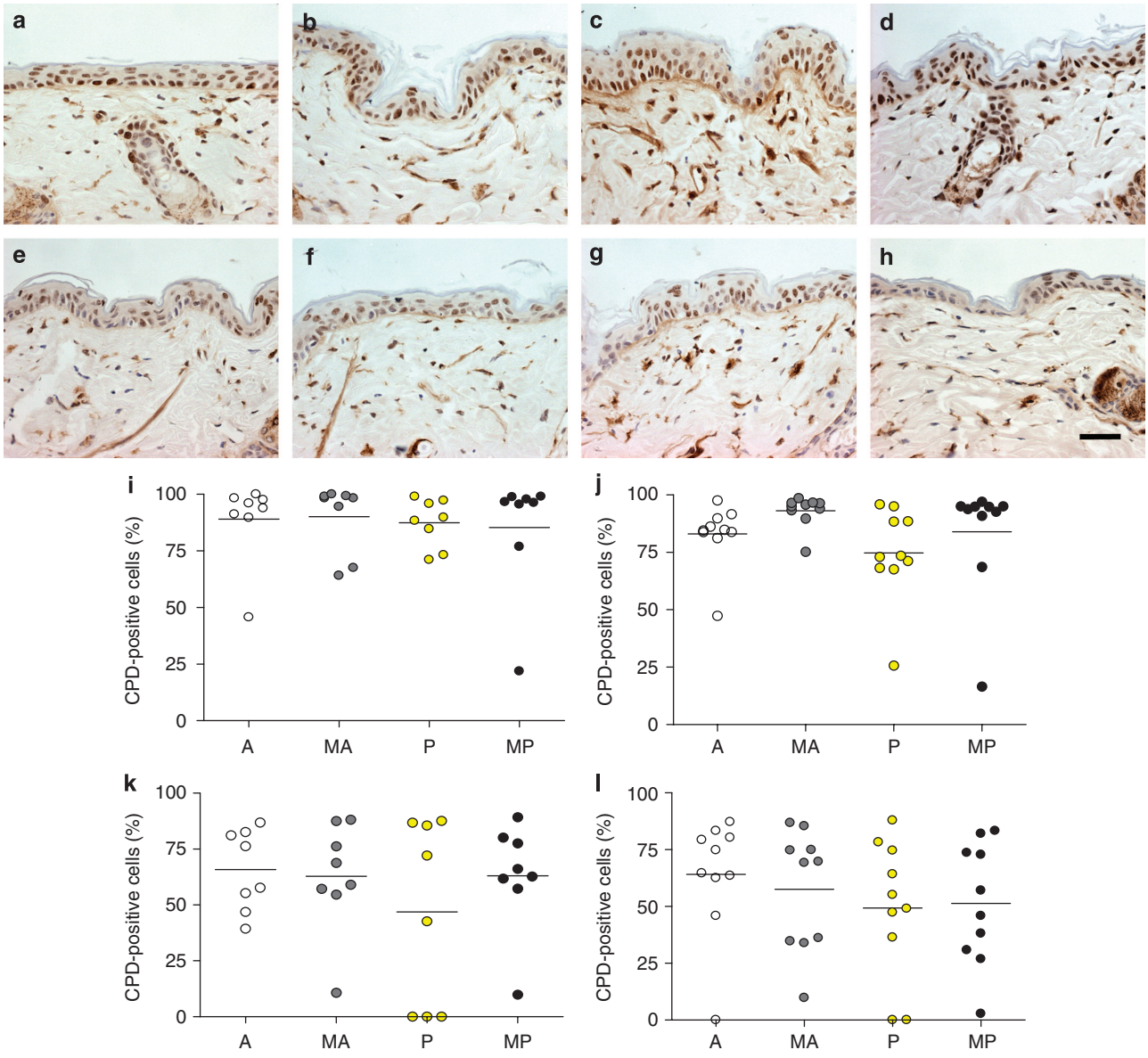


**Figure 3. Effect of single dose of 0.5 minimal edema dose (MEdD) UVR on production and early repair of CPDs in skin from A, MA, P, and MP mice.** (**a–h**) Cyclobutane pyrimidine dimers (CPDs) in dorsal skin from naive mice 30 minutes after UVR (**a–d**), with weaker staining at 24 hours after UVR (**e–h**) in (**a**, **e**) *Mc1r*$^{-/-}$ albino (A), (**b**, **f**) *MC1R*$^{+}$*Mc1r*$^{-/-}$ albino (MA), (**c, g**) *Mc1r*$^{-/-}$ pigmented (P), and (**d**, **h**) *MC1R*$^{+}$*Mc1r*$^{-/-}$ pigmented (MP) mice. Scale bar = 20 μm. (**i, j**) There were no differences between the groups in epidermal CPD-positive nuclei in irradiated skin from naive mice at (**i**) 30 minutes and (**j**) 24 hours after UVR. (**k, l**) No significant differences were observed in epidermal CPD-positive nuclei in dorsal skin at (**k**) 30 minutes or at (**l**) 24 hours after the same dose of UVR had been delivered to mice acclimatized to UVR. Points in **i–l** represent values from individual mice with bars indicating the mean value from eight animals per group.

keratinocytes to form p53 clones, some of which subsequently develop into cancer (Bäckvall *et al.*, 2004; Kramata *et al.*, 2005; Rebel *et al.*, 2005). The development of p53 clones therefore serves as a sensitive assay to measure inadequate repair of UVR-induced DNA damage in single epidermal cells and for effects on clonal proliferation in skin.

After repeated UVR irradiation for 6 weeks, epidermal sheets were prepared from the mid-dorsal skin ($n = 8$ mice per group). Clones of p53-positive cells were observed in all four sets of animals, but their frequency per unit area differed between the groups (Figure 5). Significantly lower numbers of p53-positive clones were observed in MA than A mice ($P < 0.05$), suggesting that MC1R offers non-pigmentary protection against UVR *in vivo*. In addition, fewer p53 clones were detected in P than A mice ($P < 0.05$), consistent with protection by melanin against UVR *in vivo* despite the absence of MC1R. The least p53 clone numbers were observed in MP mice ($P < 0.001$, MP vs A groups), presumably due to the combination of protective effects by MC1R and melanin. Because a subsequent UVR-induced genetic event might allow development of skin cancer

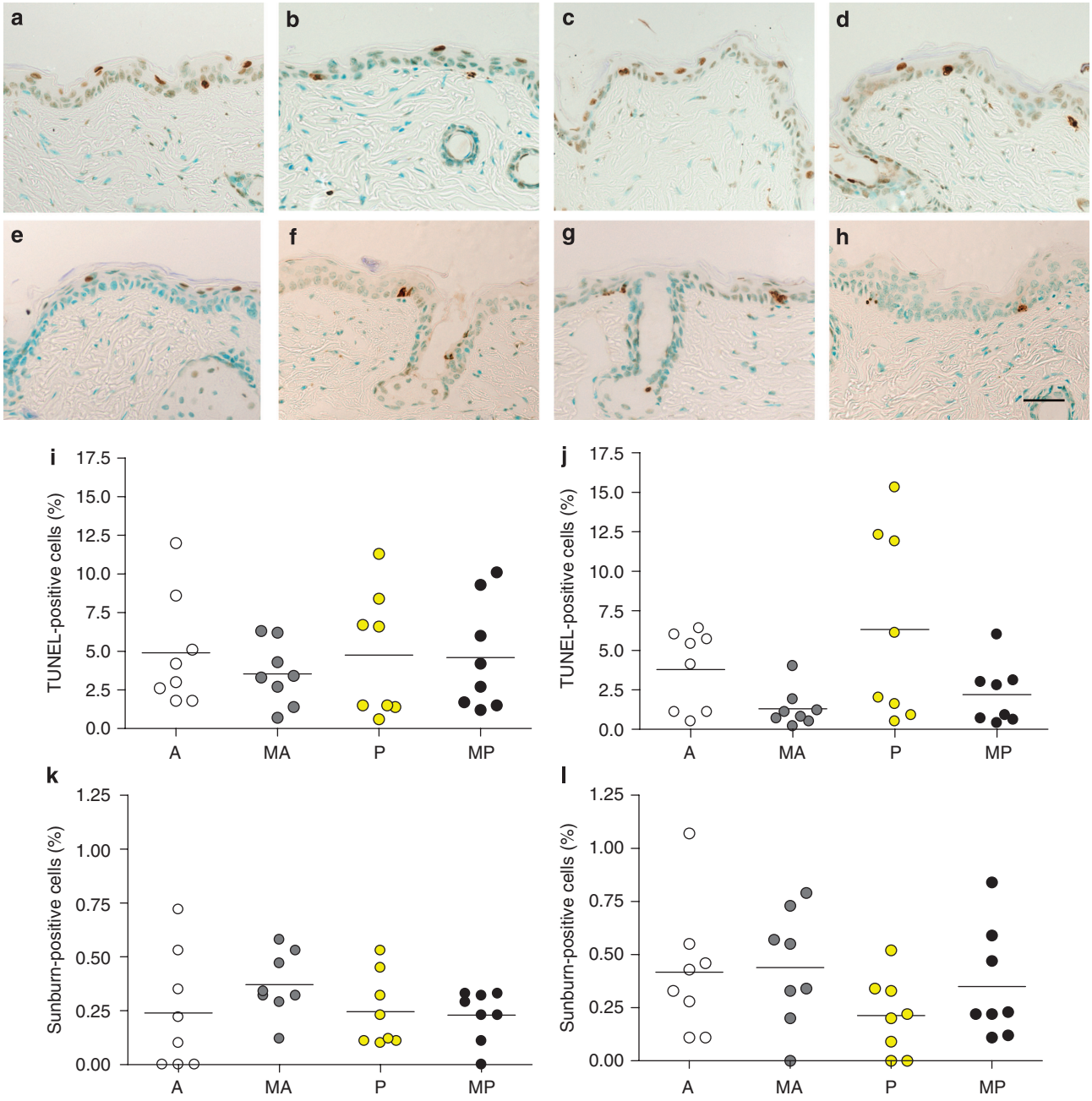


**Figure 4. Effect of single dose of 0.5 minimal edema dose (MEdD) UVR on cell death at 24 hours in skin from A, MA, P, and MP mice.** (**a**–**j**) Dorsal skin from naive (**a**–**d**) and UVR-acclimatized (**e**–**h**) mice stained for TUNEL in (**a**, **e**) *Mc1r*$^{-/-}$ albino (A), (**b**, **f**) *MC1R*$^{+}$*Mc1r*$^{-/-}$ albino (MA), (**c**, **g**) *Mc1r*$^{-/-}$ pigmented (P), and (**d, h**) *MC1R*$^{+}$*Mc1r*$^{-/-}$ pigmented (MP). TUNEL positive cells (TPCs) are stained brown and nuclei stained green. Scale bar = 20 μm. There were no significant differences between the four groups in the percentages of TPCs in (**i**) naive and (**j**) acclimatized mice. (**k**–**l**) There were fewer sunburn cells (SBCs) than TPCs (SBC values were approximately 10% of TUNEL values), but no significant differences between groups in percentages of SBCs in (**k**) naive or in (**l**) acclimatized mice. Points in **i**–**l** represent values from individual mice with bars indicating the mean value from eight animals per group.

from any single cell within these clones, the total numbers of p53-positive clone nuclei per unit area was examined; fewer positive p53 nuclei were present in the MA ($P<0.05$), P ($P<0.01$), and MP ($P<0.001$) than in A mice. Positive clones were dissected from epidermal sheets from mice in each group, and sequenced for exons 5–9 of the p53 gene. Mutations were detected in 10 of 21 (48%) clones sequenced at these five exons, with a second mutation identified in two clones; 10 were missense mutations, and included Arg178His (1 case), Ile248Asn (1 case), Leu254Gln (4 cases), Leu262Pro (1 case), Cys272Gly (1 case), Gly299Val (1 case), and Ala311Pro (1 case). Mutations were observed in each mouse group and were present at dipyrimidine sites in six clones, consistent with causation by UVR.

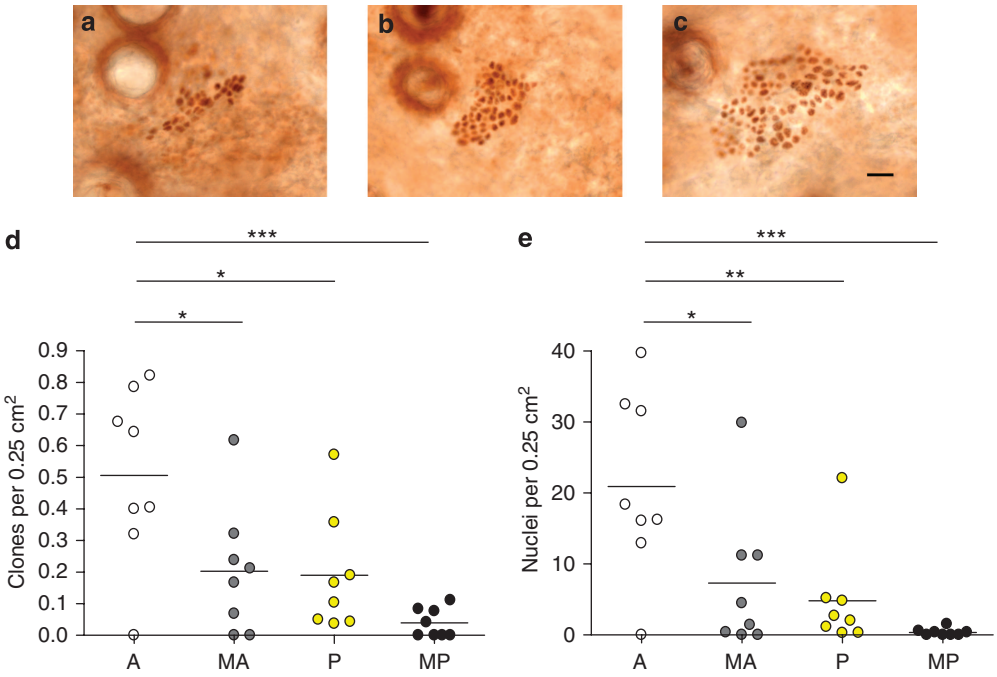


**Figure 5. Formation of p53 clones after repeated irradiation with UVR for 6 weeks.** Epidermal sheets were immunostained at 72 hours after the last irradiation for p53. (**a**–**c**) Examples of p53 clones in dorsal skin. Scale bar = 20 μm. (**d**) Significantly fewer clones were detected in $MC1R^{+}Mc1r^{-/-}$ albino (MA), $Mc1r^{-/-}$ pigmented (P), and $MC1R^{+}Mc1r^{-/-}$ pigmented (MP) than in $Mc1r^{-/-}$ albino (A) mice. (**e**) The total number of p53-positive epidermal nuclei (which is a combination of clone size and frequency) were significantly lower in MA, P, and MP than in A mice; $*P<0.05$, $**P<0.01$, $***P<0.001$; points in **d** and **e** represent values from individual mice with bars indicating the mean value from eight animals per group.

## DISCUSSION

The evidence from studies on human skin and pigmented mouse models indicates that epidermal melanin protects against UVR (Yamazaki *et al.*, 2004; Yamaguchi *et al.*, 2006; Kato *et al.*, 2007). However, it is important to understand how MC1R photoprotects, and the role of melanin type in relation to the detrimental effects of UVR to make balanced decisions about UVR exposure in terms of DNA damage and potential benefits from cutaneous vitamin D synthesis (Moan *et al.*, 2008). This study shows that melanin type and MC1R do not exert a major influence on the generation of CPD, and their early repair, in skin after single UVR exposures. Takeuchi *et al.* (2004) similarly noted no differences in the CPD responses of hair-bearing skin of black, yellow, and albino mice to a single UVR dose. These observations are not surprising because human non-melanoma skin cancers generally develop after multiple UVR exposures, consistent with minor rather than major differences in DNA damage/repair being responsible for individual susceptibility. The effects of chronic repeated UVR differ within and between human populations, and the current investigations on p53 clones help clarify the issues concerning the non-pigmentary MC1R-mediated effects and the role of melanin in this scenario. Although p53 gene mutations were not identified in all clones, the proportion with mutations is similar to other studies and consistent with UVR-induced somatic mutations/alterations in p53 permitting subsequent preferential proliferation of these cells during repeated UVR exposure.

The reduced p53 clone numbers in $MC1R^{+}$ albino mice compared with albino mice without MC1R indicate that MC1R has protective effects *in vivo*, which cannot solely be accounted for by melanin type or amount, and that MC1R-mediated non-pigmentary protection is a substantial component of the overall protection afforded by this receptor. In conjunction with the *in vitro* work by Böhm *et al.* (2005) and Kadekaro *et al.* (2005), it seems likely that incomplete repair of UVR-induced DNA damage in individual cells lacking fully functional MC1R, coupled to a lower level of apoptosis, leads to subsequent clonal expansion of these mutated cells *in vivo*. In addition, based on the fact that gene arrays of neonatal skin from pigmented mice lacking Mc1r indicated that the Mc1r-dependent UVB response involves genes that regulate cell cycle and oncogenesis (April and Barsh, 2007), it is possible that these MC1R-mediated mechanisms helped to protect partially against p53 clone development in the $MC1R^{+}$ mice in this study. In the human skin cancer studies, *MC1R* variants may have remained a risk factor for cutaneous neoplasia after controlling for skin type/hair color simply as an artifact of the well-recognized limitations of skin typing (Rampen *et al.*, 1988; Bastiaens *et al.*, 2001; Box *et al.*, 2001; Landi *et al.*, 2005; Liboutet *et al.*, 2006). However, this work supports the interpretation in those studies that MC1R has non-pigmentary mechanisms that protect against UVR *in vivo*.

A phototoxic role of pheomelanin and a role for this melanin type in UVR carcinogenesis have been suggested mainly from *in vitro* research (Chedekel *et al.*, 1978; Harsanyi *et al.*, 1980; Menon *et al.*, 1983; Wenczl *et al.*, 1998). Human skin contains eumelanin and pheomelanin (Thody *et al.*, 1991), therefore investigations using eumelanin/

pheomelanin ratios similar to those in human skin are more helpful in dissecting out phototoxicity from protective effects. Furthermore, although red hair melanin is more photomutagenic than black hair melanin, the evidence for pheomelanin toxicity has often been suggested from studies using cell death as readout (Harsanyi *et al.*, 1980; Menon *et al.*, 1983; Takeuchi *et al.*, 2004). In this study the fewer number of p53 clones in the pigmented mice than in the albino mice lacking MC1R suggests that the melanin type in fair-skinned individuals would partially protect against chronic repeated UVR exposure. This lack of greater damage in the pigmented mice without MC1R is in keeping with the view of Hennessy *et al.* (2005) who speculated that, based on their melanin results in human skin, factors other than pheomelanin may be important in determining UV susceptibility in red-haired fair-skinned individuals. It is possible that, based on the work by Takeuchi *et al.* (2004), UVR may have phototoxic effects within hair follicles in red-haired humans, but it is unclear whether this might be responsible for (part of) the higher skin carcinogenesis in human redheads. In the case of interfollicular skin (and probably also in follicular skin containing vellous rather than terminal hairs), our results suggest that fair-skinned people are likely to be partially protected from UVR by their cutaneous melanin. Further support for this comes from the fact that albino subjects in Africa seem to develop non-melanoma skin cancers more commonly and at a younger age than fair-skinned Caucasians who reside for similar periods of time in similar UVR environments (Luande *et al.*, 1985; Buettner and Raasch, 1998).

The results of this study indicate that MC1R protects against UVR by a combination of pigmentary and non-pigmentary mechanisms *in vivo*. Furthermore, they suggest that fair-skinned individuals are at a greater risk of the detrimental effects of UVR, including skin cancer development, principally because of a reduction in photoprotection by melanin and a lack of MC1R-mediated non-pigmentary protection. Moreover, the results signify that the consequences of pheomelanin in fair skin need to be viewed in the context of the eumelanin/pheomelanin ratio, which based on this study suggests that the overall effects of pheomelanin in fair skin are not detrimental.

## MATERIALS AND METHODS

### Generation of *in vivo* hairless MC1R model

Albino Skh:hr-1 hairless mice (*EEcchh*, in which *E/e*, *C/c*, and *H/h* represent extension/Mc1r, tyrosinase, and hairless loci, respectively) were crossed with pigmented $MC1R^{+}Mc1r^{-/-}$ mice ($MC1R^{+}$ *eeCCHH* (recessive yellow mice transgenic for human MC1R); Healy *et al.*, 2001) and the presence of MC1R in the progeny was determined by PCR and restriction fragment length polymorphism. This $MC1R^{+}Mc1r^{-/-}$ approach was taken, rather than simply using mice null for murine *Mc1r*, because it was considered that the final model would be more informative about the pigmentary and non-pigmentary effects of human MC1R. $MC1R^{+}$ *EeCcHh* mice were backcrossed with Skh:hr-1 and *hh*, *cc*, and *Cc* offspring determined by hairless, albino, and pigmented phenotypes and confirmed by PCR and restriction fragment length polymorphism. Murine *Mc1r* status (*EE/Ee*) was determined by PCR/sequencing. Unrelated *EeCchh* and *Eecchh* mice (one of each pair $MC1R^{+}$) were intercrossed, and subsequently *eeCchh* and *eecchh* animals (one $MC1R^{+}$) were intercrossed for successive generations to produce the MC1R hairless model in which A, MA, P, and MP animals were present in each litter. All experiments were carried out under licenses held under the Animals (Scientific Procedures) Act and with approval by the University of Southampton ethical committee for research involving animals.

### Measurement of stratum corneum and epidermal thickness

Images were taken of hematoxylin and eosin, 4 μm paraffin sections of dorsal skin on a Zeiss Axioscope-2 microscope/Axicam camera system (Welwyn Garden City, UK) ( × 40). Measurements of stratum corneum and nucleated epidermis were taken at 10 random points in each of three images per animal, six animals per group, using Adobe Photoshop (Uxbridge, UK); the mean age of each group was 9.5 weeks (naive mice) and 10–12 weeks (acclimatized mice).

### Melanin assays

Dorsal skin epidermis was split from dermis by overnight incubation in 2 U dispase (Sigma-Aldrich, Dorset, UK) and freeze dried. Eumelanin and pheomelanin levels were quantified by chemical degradation; eumelanin by acidic permanganate oxidation to form pyrrole-2,3, 5-tricarboxylic acid, and hydriodic acid reductive hydrolysis of pheomelanin to form 4-amino-3-hydroxyphenylalanine. Concentrations of these products were analyzed by high-performance liquid chromatography and results were converted to eumelanin and pheomelanin concentrations by multiplying 50 times for eumelanin and 9 times for pheomelanin (Wakamatsu and Ito, 2002). Paraffin sections (4 μm) were stained for eumelanin by the Masson-Fontana method.

### UVR irradiation protocols and sample preparation

Irradiations were carried out under Arimed B lamps (Cosmedico, Stuttgart, Germany) emitting UVA (320–400 nm) and UVB (280–320 nm) in a ratio similar to midday summer sunlight (UVA 96% and UVB 4%). After MEdD determination on naive skin, equivalent to 10.3 kJ/m$^{-2}$, the animals were irradiated with single doses of 0.5 and 2 MEdD, with mice alert and allowed to move freely and with no more than four mice per cage. Dorsal skin samples were obtained 30 minutes and 24 hours later, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 hours, and embedded in paraffin. Repeated UVR irradiations were administered at 0.2 MEdD per day, five days weekly for 6 weeks. After 4 days,, mice received a single UVR dose of 5.15 kJ m$^{-2}$ or 20.6 kJ m$^{-2}$ and dorsal skin samples were obtained at 30 minutes and 24 hours. In p53 clone experiments, animals were irradiated for 6 weeks as above, and dorsal skin was obtained for epidermal sheets at 3 days after final UVR dose. Epidermal sheets were separated from dermis after incubating skin samples in 20 mM EDTA in PBS for 2–3 hours at 37 °C, and then fixed in acetone at 4 °C for 20 minutes, rinsed in PBS, and stored at 4 °C in PBS.

### Immunohistochemistry of paraffin-embedded skin sections

CPDs were detected in 4 μm paraffin-embedded sections using CPD-specific antibody TDM-2 (kind gift from O. Nikaido, Japan). Sections were dewaxed, endogenous peroxidise blocked with 0.5% $H_2O_2$ in methanol, washed with PBS, and the epitope unmasked with 1% pronase in PBS (Sigma-Aldrich, UK) followed by incubation with

0.07 M NaOH in 70% ethanol. Nonspecific binding was blocked with avidin/biotin blocking solution (Vector Laboratories, Peterborough, UK) followed by PBS/20% fetal bovine serum/1% BSA. Sections were incubated overnight in a humidified chamber at 4 °C with TDM-2 antibody, 1:4,000 in PBS. After washing with PBS, sections were incubated for 30 minutes at room temperature with biotin-conjugated rabbit anti-mouse antibody solution (Dako, Glostrup, Denmark) diluted 1:200. Staining was visualized using an ABC system (Dako, Denmark) and diaminobenzidine (Biogenex, San Ramon, CA). Sections were counterstained with Mayer's Hematoxylin. CPD-positive epidermal nuclei were counted under microscopy using standardized conditions and results were expressed as percentage of epidermal cells that were positive; at least 400 epidermal cells were counted per animal, with eight animals per group. Intensity of stain was also scored on a 1–5 scale, in which 1 equals no staining and 5 maximal staining.

### Apoptosis assays

SBCs were identified by light microscopy in hematoxylin and eosin sections of dorsal skin by their characteristic morphology: condensed, pyknotic, darkly basophilic nuclei, eosinophilic cytoplasm, and intercellular gap (halo) formation (Sheehan and Young, 2002). Between 850 and 1,050 interfollicular epidermal cells were examined in eight mice per group, and results were expressed as percentage of epidermal cells that were SBCs. TUNEL staining was performed using an ApopTag peroxidase *in situ* apoptosis detection kit according to the manufacturer's instructions (Chemicon Chandlers Ford, Hampshire, UK) with diaminobenzidine as substrate and 0.5% methyl-green as nuclear counterstain. Interfollicular and follicular epidermal cells were examined, with 400–500 cells counted in each area per animal, eight animals per group, and results were expressed as percentage of epidermal cells that stained positive.

### Immunostaining of epidermal sheets

Fixed epidermal sheets were stained for p53 clones or melanocytes. Epitopes were unmasked by microwaving for 10 minutes in 10 mM citrate buffer (pH 6.0) followed by PBS/0.05% Tween washes. Endogenous peroxidase was blocked by 0.5% $H_2O_2$ in methanol for 20 minutes, the sheets were washed with PBS/0.05% Tween and nonspecific antibody binding was blocked with PBS/20% fetal bovine serum/1% BSA for 1 hour at room temperature. Samples were incubated overnight in PBS/20% fetal bovine serum/1% BSA containing 1:500 CM5 antibody (Novocastra Laboratories, Newcastle upon Tyne, UK) for p53 or 1:200 αpep1 (kind gift from V. Hearing) for tyrosinase-related protein-1 at 4 °C. After PBS/0.05% Tween washes, sheets were incubated with 1:400 biotin-conjugated swine anti-rabbit immunoglobulin (Dako, Denmark) followed by ABC complexes (Dako, Denmark) and diaminobenzidine. Sheets were mounted in DPX mountant for visualization.

### p53 clone frequency

Immunostained epidermal sheets were blinded and analyzed by light microscopy, ×200 magnification with an eyepiece graticule, to allow systematic coverage of the entire sheet. p53 clones were identified as clusters of ⩾3 brown staining nuclei. Number of p53-positive clones/unit area and number of cells per clone were determined by varying the focus to observe the different cell layers; at least 10 grids (each 0.25 $cm^2$) were counted per animal.

### Clone microdissection, DNA amplification, and sequencing

p53 clones were isolated from epidermal sheets (immunostained using diaminobenzidine and left unmounted in PBS) using individual 30-gauge needles under an inverted light microscope. DNA was isolated by incubating samples overnight at 65 °C in 10 mM Tris-HCL (pH 8.5), 1% Tween 80, and 3.2 μg $μl^{-1}$ proteinase K (Calbiochem, Nottingham, UK) buffer, before proteinase K was inactivated at 95 °C for 15 minutes and samples used for PCR. Specific p53 PCR primers for exons 5/6 (5′-CAGTCCTCTCTTTGCTGG-3′, 5′-GCCTAGCTAG CACTCAGG-3′), 7 (5′-GCCGAACAGGTGGAATATCC-3′, 5′-CCCA CCTGTTCCCAACCC-3′), and 8/9 (5′-GACGTCTCTTATCTGTG GC-3′, 5′-GAGACAGAGGCAATAATGGG-3′) gave 627 bp, 492 bp, and 512 bp amplicons, respectively. All three regions were amplified using Biotaq DNA polymerase (Bioline, London, UK), 1 × Optibuffer (Bioline), 1 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, and relevant primers in a Perkin Elmer Cetus 9700 thermal cycler (Beaconsfield, UK), with a denaturation cycle at 94 °C for 5 minutes, then 35 cycles at 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute, and a final extension at 72 °C for 7 minutes. Amplicons were visualized on 1% agarose gels stained with ethidium bromide and bands were excised and purified using QIAquick gel extraction kit (Qiagen, Crawley, UK). Sequencing was performed with a DTCS Quick Start sequencing kit (Beckman Coulter, High Wycombe, UK) on a CEQ8800 Genetic Analysis System (Beckman Coulter) using specific primers for exons 5/6 (5′-CCTCTGCCACTGCGAGGG-3′), 7 (5′-ACTG AAATTATTAGAGG-3′), and 8/9 (5′-TCGGGGTTCCTGTAACTGG-3′).

### Statistics

Statistical significance of eumelanin/pheomelanin ratios was analyzed using unpaired *t*-test (one tailed was used because previous research indicates this ratio would be higher with MC1R present). Significance of p53 clones was assessed using one-way analysis of variance with a Bonferroni multiple comparison test (Graphpad statistical package, La Jolla, CA).

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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